Rif1-Dependent Regulation of Genome Replication in Mammals

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Eukaryotic genomes are replicated starting from multiple origins of replication. Their usage is tightly regulated and not all the potential origins are activated during a single cell cycle. In addition, the ones that are, are activated in a sequential order. Why don't origins of replication normally all fire together? Is this important? And if so, why? Would any order of firing do, or does the specific sequence matter? How is this process regulated? These questions concern all eukaryotes, but have proven extremely hard to address because replication timing is a process intricately connected with multiple aspects of nuclear function.

In 2012 the identification of the first genome-wide regulator of replication timing across evolution, a protein called Rif1 (Cornacchia et al., 2012; Hayano et al., 2012; Yamazaki et al., 2012), provided a long-awaited tool to start addressing these questions. However, Rif1 itself has also proven a very complex protein, confusingly involved in telomere length control (Gallardo et al., 2011; Hardy et al., 1992; Teixeira et al., 2004), DNA repair (Buonomo et al., 2009; Chapman et al., 2013; Daley and Sung, 2013; Feng et al., 2013; Martina et al., 2014), DNA replication timing (Cornacchia et al., 2012; Dave et al., 2014; Hayano et al., 2012; Hiraga et al., 2014; Mattarocci et al., 2014; Peace et al., 2014; Sreesankar et al., 2015; Yamazaki et al., 2012) and nuclear architecture organization (Foti et al., 2016). Thus, the complexity of Rif1 biology reflects the intricacies of replication-timing control at a molecular level.

Rif1 and DNA replication timing

Rif1 was originally discovered in budding yeast as a negative regulator of telomere length, where it is brought to the telomeric repeats through its interaction with Rap1 (Hardy et al., 1992), a sequence-specific DNA-binding protein that recognizes and directly binds telomeric repeats in yeast (Conrad et al., 1990; Lustig et al., 1990). Hence, the amount of Rif1 at telomeres is proportional to telomere length (Levy and Blackburn, 2004). Interestingly, both sudden telomere shortening (Bianchi and Shore, 2007) and Rif1 deletion-induced telomere elongation (Lian et al., 2011) correlate with a shift of telomere replication from late- to early S-phase. While it was originally hypothesized that telomere length *per se* might have affected the timing of firing of subtelomeric origins, these data suggested instead that Rif1 presence at budding yeast telomeres was responsible for their late replication (Hiraga et al., 2014). In mammalian cells, Rif1 is not detectable at normal telomeres (Silverman et al., 2004; Xu and Blackburn, 2004), nor does it seem to be involved in telomere length regulation (Buonomo et al., 2009). This is consistent with the fact that mammalian telomeres do not specifically replicate in late S-phase (Arnoult et al., 2010; Hultdin et al., 2001; Wright et al., 1999; Zou et al., 2004). Rif1 deficiency induces genome-wide changes in replication timing in *Schizosaccharomyces pombe* (Hayano et al., 2012), *Saccharomyces cerevisiae* (Peace et al., 2014), *Drosophila* (Sreesankar et al., 2015), mouse and human cells (Cornacchia et al., 2012; Yamazaki et al., 2012). Hence, Rif1-dependent control of replication timing is highly conserved throughout evolution.

In yeast, the control of replication timing via Rif1 requires its interaction with the Ser/Thr phosphatase PP1 (Dave et al., 2014; Hiraga et al., 2014; Mattarocci et al., 2014). Disruption of this interaction increases the amount of phosphorylated MCM4, thereby potentially promoting origin firing (Fu et al., 2011). According to the model drawn on the basis of these results, during the G1/S transition and the initial part of S-phase Rif1 recruits PP1 to the origins destined to fire later in S-phase, acting locally to counteract the activating phosphorylation of MCM4 by Dbf4-dependent kinase (DDK). This hypothetical pathway places Rif1-dependent regulation of origin firing at the stage of execution of the replication-timing program. Recent data obtained also from human cell lines (Alver et al., 2017; Hiraga et al., 2017) support this hypothesis. However, there are also experimental results suggesting that there could be more to Rif1 function. The overlap between Rif1-bound origins and late/dormant is modest in yeast (Hayano et al., 2012; Peace et al., 2014), and in mouse embryonic stem cells (ESCs) there is no clear enrichment of Rif1 at the limited number of replication origins analyzed (Foti et al., 2016). This is puzzling in light of the model that sees Rif1 functioning locally at late origins as a PP1 adaptor, suggesting the possibility that Rif1 could control origin activation through additional mechanisms, acting on a genome-wide scale. In mammalian cells, there is evidence that this could be the case. Below, I am going to discuss some of the relevant data, and what it tells us about Rif1's role and the relationship between nuclear organization and regulation of replication timing.

Rif1, nuclear organization and replication timing

The idea that spatial nuclear organization and sequential firing of replication origins are connected relies on evidence accumulated over the past several years. For example, based on the frequency of intra-domain chromatin interactions, the mammalian nucleus has been subdivided into two large compartments, A and B (Lieberman-Aiden et al., 2009), displaying a striking correlation with early and late replicating fractions of the genome (Ryba et al., 2010; Yaffe et al., 2010). More recently, it has been shown that the units that constitute the building blocks of these large nuclear compartments, the topologically associated domains (TADs) (Dixon et al., 2012), coincide with the units of replication-timing regulation, the developmentally defined replication domains (Pope et al., 2014). At the molecular level, it has recently been shown in budding yeast that Fkh1 and Fkh2 promote early firing of origins by mediating their clustering (Knott et al., 2012). On the other hand, the relationship between DNA localization at the nuclear periphery and its late replication is well established, with developmentally regulated loci switching replication from late to early S-phase (LtoE) while also relocating towards the internal part of the nucleus and vice versa (Hiratani et al., 2010; Hiratani et al., 2008; Williams et al., 2006; Zhou et al., 2002). Similarly, the time at which replication timing is re-established after mitosis (timing decision point, TDP), in early G1, coincides with chromosome re-positioning, when domains destined to be late replicating occupy the nuclear periphery and peri-nucleolar areas (Dileep et al., 2015; Dimitrova and Gilbert, 1999; Raghuraman et al., 1997). The peripheral position of late replicating domains is conserved throughout evolution.

Rif1 localizes to the nuclear periphery in a variety of cells, from yeast to mammals. In mammals roughly half of the pool of Rif1 is associated with the nuclear periphery and interacts with the lamina (Cornacchia et al., 2012; Foti et al., 2016; Roux et al., 2012; Yamazaki et al., 2012). In addition, Rif1 is distributed in large domains (Rif1 associated domains or RADs) strikingly overlapping with the late replicating genome (Foti et al., 2016). This distribution is highly reminiscent of the genome-binding profile of one of the major components of the nuclear lamina, Lamin B1. The nuclear lamina plays an important role in the organization of nuclear architecture (reviewed in (Shimi et al., 2010)). Lamin B1 covers large domains called Lamin B1-associated domains (LADs) (Peric-Hupkes et al., 2010), that also overlap with the late replicating genome (Peric-Hupkes et al., 2010), although not as extensively as RADs (our unpublished results). Finally, LADs and RADs display a substantial degree of overlap. Overall, these data suggest that Rif1 could have a role in three-dimensional (3D) organization of the genome in the nucleus. In agreement with this hypothesis, Rif1 deficiency in mammalian cells has been shown to alter chromatin loop size (Yamazaki et al., 2012), chromocenter compaction (Cornacchia et al., 2012) and induce loss of temporal coordination of the replication of neighboring as well as more distantly interacting replicons (Cornacchia et al., 2012; Foti et al., 2016). Nevertheless, these effects could be consequential to the changes of replication timing. However, we have recently shown that deletion of Rif1 in primary mouse embryonic fibroblasts arrested in G0 impacts the 3D organization of replication domains as early as the first G1 after Rif1 deletion (Foti et al., 2016). Such early alterations of chromatin architecture might be responsible for the changes in replication timing that appear in the following S-phase. In fact, the constrains that normally limit domain interactions within either early or late replicating domains (Takebayashi et al., 2012) are lost in the absence of Rif1, with late domains promiscuously establishing numerous, low frequency contacts with early domains and vice versa (Foti et al., 2016) (Fig. 1 A and B). In agreement with this, we have observed by locus-specific 3D FISH that LtoE switches induced by Rif1 deletion are also accompanied by a tendency to re-locate the corresponding portions of the genome from the periphery to the interior of the nucleus (our unpublished data). These observations indicate that in G1, Rif1 plays a role in the organization of nuclear architecture, possibly at two different levels: it orchestrates and spatially constrains contacts between different replication domains, and ensures peripheral positioning of at least some late chromosomal domains. We suggest that these functions of Rif1 in nuclear architecture could be a first tier at which Rif1 instructs replication timing, as both of these aspects of nuclear organization have been related to replication timing.

Rif1 and PP1

In line with the identification of yeast Rif1 as a mediator of PP1 function at replication origins, mammalian and *Drosophila* Rif1s were identified in PP1-associated complexes (Guruharsha et al., 2011; Moorhead et al., 2008; Trinkle-Mulcahy et al., 2006). In addition, we have obtained structural and functional data classifying mouse Rif1 as a *bona fide* PP1 regulatory subunit (Sukackaite et al., 2017).

It is therefore clear that one Rif1 function conserved throughout evolution is to act as a regulatory subunit of PP1 (Sreesankar et al., 2012). However, the idea that Rif1 could play a role in G1 during the establishment of replication timing is not mutually exclusive with its proposed function at late origins in G1/S. The spatial sequestration of late origins away from early ones (and from DNA replication's limiting factors? (Mantiero et al., 2011; Patel et al., 2006; Tanaka et al., 2011; Wu and Nurse, 2009)) through the formation of RADs would also result in creation of large areas of high PP1 concentration which could facilitate PP1 function on late origins later in the cell cycle. In this scenario, Rif1 would act both in early G1 at the level of establishment (Fig. 2A and B) and later, at G1/S transition, at the execution step of the replication-timing program (Fig. 2C).

Alternatively, it is formally possible that the architectural function of Rif1 has no bearing on replication timing *per se* and that the common denominator between nuclear 3D organization and replication timing is Rif1 as a molecule, independently involved in both processes. However, the loss of replication timing specific domain contacts observed in G1 in Rif1 null cells seems to contradict this hypothesis (Fig. 3).

Finally, direct transposition of functional data between distant organisms can be misleading. It cannot be excluded that in metazoa Rif1 has evolved an architectural function that is absent or not essential in yeast. This change could indeed have taken place concomitantly with the transition from a closed to an open mitosis, the evolution of the lamina and the need to re-create an ordered nucleus after each mitosis (Sazer et al., 2014). In fact, it is not known whether the lamina or other nuclear architectural components also play a role in either the establishment or execution of the DNA replication-timing program. However, some preliminary observations suggest that they could. We have found that replication-timing regulation of the late replicating genome is indeed differentially sensitive to Rif1 function, depending on the concomitant stable presence of Lamin B1. The regions of the late replicating genome concurrently associated with Rif1 and Lamin B1 (RADs-LB$^+$) do not change their replication timing upon Rif1 deletion. In contrast, late replication of genomic regions associated with Rif1 only (RADs-LB$^-$) is entirely Rif1 dependent (Foti et al., 2016) (Fig. 1A and B). We have therefore unexpectedly revealed a further level of complexity in the regulation of replication timing, once again coinciding with some aspects of nuclear architecture organization. This finding also reinforces the question of the role of PP1 in Rif1-mediated control of replication timing. If PP1 is indeed the sole, ultimate effector of Rif1 function, another protein has to be responsible for recruiting PP1 at RADs-LB$^+$ in Rif1 null cells. This is in principle possible, as the PP1 phosphatase has been identified as a partner of different proteins associated with the nuclear envelope, such as AKAP149 (Steen et al., 2000) and LAP1β (Santos et al., 2013).

Telomere length regulation, DNA repair, replication timing and nuclear organization: where is the connection?

Rif1 has been involved in processes other than replication timing and organization of nuclear architecture, namely DNA repair (Buonomo et al., 2009; Chapman et al., 2013; Daley and Sung, 2013; Feng et al., 2013; Martina et al., 2014) and telomere length regulation (Gallardo et al., 2011; Hardy et al., 1992; Teixeira et al., 2004). Such diversity of roles is puzzling, and could either reflect multiple independent functions of different parts of this very large protein, or multiple outcomes of a single molecular property of the protein applied in different contexts, or both.

The understanding of the functional organization of Rif1 domains is still rudimentary. The N-terminus is highly conserved, composed of numerous HEAT repeats (Silverman et al., 2004; Sreesankar et al., 2012) required for localizing the protein to double strand breaks in mouse cells, through binding of phosphorylated 53BP1 (Escribano-Diaz et al., 2013). The large middle region is non-conserved and predicted to be a non-structured polypeptide. Interestingly, in both mouse and human Rif1 genes this region is encoded by a single, ~3000bp long exon, accounting for almost half of the entire coding region. This unusual gene organization might suggest acquisition of a mammalian-specific function. The C-terminus of Rif1 contains three recognizable domains, indicated as conserved regions C, of which CI and CII are present from yeast to humans, while CIII is only conserved within vertebrates. In metazoa, CI harbors PP1 interacting motifs, which are located at the N-terminus of yeast Rif1. CII is a very intriguing portion of the protein. In metazoa it contains a DNA binding domain, preferentially recognizing cruciform structures (Sukackaite et al., 2014; Xu et al., 2010). However, the relevance of this interaction with DNA *in vivo* is yet to be determined, as Rif1 association with insoluble nuclear fractions renders this technically challenging to assess. Moreover, the *in vivo* distribution of cruciform DNA is unclear, having been associated with promoter melting, activated origins of replication and DNA recombination intermediates (Brazda et al., 2011). *In vivo* DNA binding has been shown for fission yeast Rif1, which recognizes a consensus sequence capable of forming G-quadruplex (G4) (Kanoh et al., 2015). However, the specific domain mediating this interaction is yet to be mapped. A putative DNA-binding domain seems to be present in the budding yeast Rif1, but not in CII (Sreesankar et al., 2012), which is instead required for interaction with Rap1 (Shi et al., 2013), DDK (Hiraga et al., 2014) and for Rif1's tetramerization (Shi et al., 2013). The residues involved in the tetramerization of *S. cerevisiae* Rif1 largely overlap with the positions implicated in DNA binding in the human and mouse homologs, suggesting the possibility that the two functions are related. Multimerization has also been shown for the mammalian protein (Xu et al., 2010), supporting the idea that Rif1 could form patches of a lamina-associated protein network anchoring RADs to the nuclear periphery.

Clearly one of the conserved properties of Rif1 is its interaction with PP1. It is therefore reasonable to hypothesize that PP1 could also mediate Rif1 functions at telomeres (Mattarocci et al., 2016) and/or during the DNA damage response (DDR), by ensuring the de-phosphorylation of telomere or DDR-specific substrates. However, at least for budding yeast telomeres, there is an alternative hypothesis

that Rif1-dependent control of late replication could be part of the mechanism directing telomerase-dependent telomere elongation. As telomerase-dependent telomere lengthening occurs after conventional replication (Diede and Gottschling, 1999), telomere replication in late S-phase would in turn limit the time available for telomerase-dependent telomere elongation (Bianchi and Shore, 2007). This mechanism could explain how telomere length equilibrium is maintained via differential replication of longer and shorter telomeres: longer telomere length would translate into higher Rif1 occupancy at that specific telomere, and higher Rif1 occupancy would impose later subtelomeric origin firing and telomere replication. Late telomere replication will result in a shorter window of opportunity for telomerase to extend that telomere within a cell cycle. Therefore, longer telomeres are replicated later and are less likely to be extended by telomerase.

The intricate politics of nuclear function

The identification of Rif1 has provided an important gateway into understanding the genetic and molecular control of replication timing. However, it has also raised numerous questions, some unexpected, and uncovered a confusing network that interconnects several fundamental nuclear functions. For example, studying the impact of Rif1 deficiency on replication timing, gene expression and nuclear organization in different cell types has revealed that nuclear architecture could be the common denominator between regulation of gene expression and replication timing. The relationship between these two aspects of nuclear function has been widely debated, as there are general correlations between genomic regions that are early replicating and those that are expressed, and, reciprocally, between late replicating regions and heterochromatic, transcriptionally repressed domains. Changes of gene expression and replication timing during development often move in the same direction, with switches of replication from early to late S-phase (EtoL) coinciding with reduced gene expression and vice versa. These data have prompted the idea that one aspect of nuclear function could determine the other. But what controls what has been challenging to determine due to contradicting data (reviewed in (Rivera-Mulia and Gilbert, 2016). Recently, a detailed analysis of replication timing and gene expression changes at different stages of human ESCs differentiation has in fact revealed that the temporal relationship between gene expression and replication timing changes is different in different regions of the genome (Rivera-Mulia et al., 2015), indicating clearly that at least one important variable is still missing from the picture. Our data indicate that the missing component could be nuclear positioning. We found that "simply" changing replication timing is not sufficient to affect gene expression. Different cell types are either permissive or not for proliferation in the absence of Rif1, probably depending on the status of the DNA damage checkpoints. For cells like mouse primary embryonic fibroblasts (pMEFs), where Rif1 deletion impairs proliferation, no gene expression changes can be detected as a consequence of the deletion, although chromatin organization and replication timing are affected (Cornacchia et al., 2012; Foti et al., 2016). However, in cells that proliferate upon Rif1 deletion, for example immortalized MEFs or ESCs, a progressive deregulation of gene expression can be

observed in time (Foti et al., 2016). These data suggest that an increasingly amplified deregulation of nuclear architecture could turn into transcriptional changes, in agreement with the evidence linking subnuclear positioning and gene expression (Andrulis et al., 1998; Finlan et al., 2008; Mattout et al., 2011; Peric-Hupkes et al., 2010; Reddy et al., 2008; Zullo et al., 2012). It remains unclear if the effect of nuclear architecture on gene expression is mediated through modification of the epigenetic landscape: we could not detect any major changes in the epigenome after Rif1 deletion, at least for the limited number of histone modifications we have examined (Foti et al., 2016).

Conclusions

The discovery of Rif1 and its multifaceted functions has represented an entry point into the molecular labyrinth of nuclear functions. Future work will have to span across different fields in order to unravel how specific to Rif1 this functional network is, or if it is a general feature of nuclear structural components. Importantly, understanding to what extent PP1 mediates different aspects of Rif1's role bears the potential of creating separation of function mutants. Integrating different aspects of nuclear function is fundamental to dissect the complexity of cellular transitions such as aging, differentiation and transformation.

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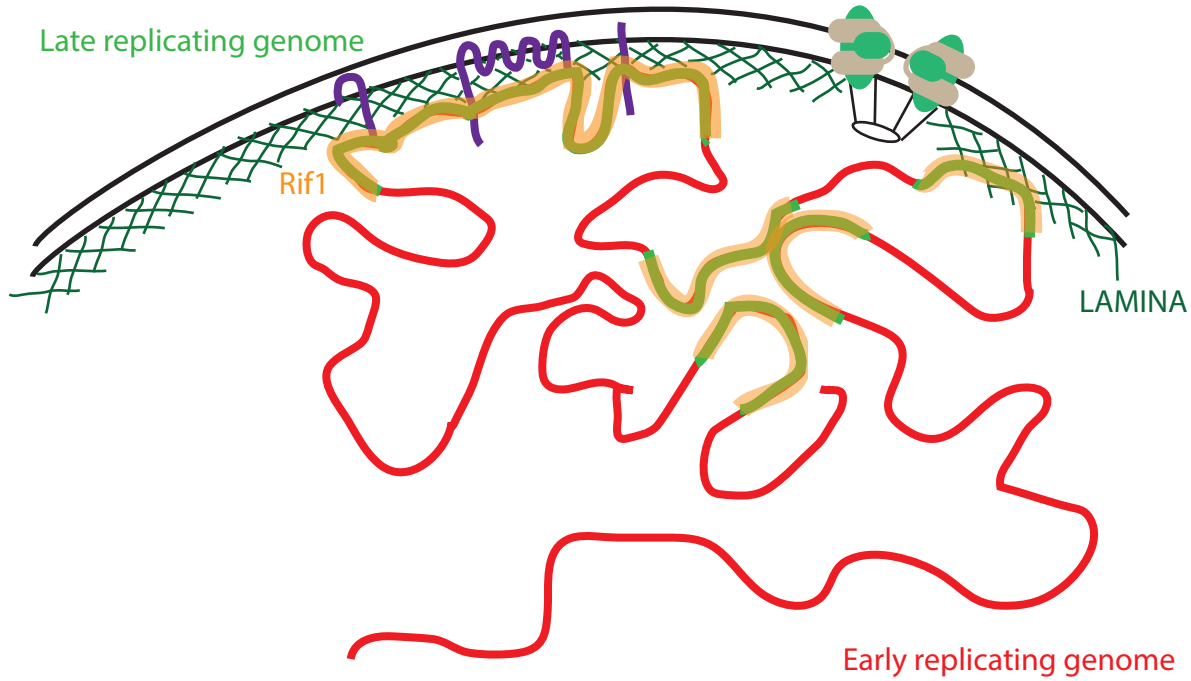
Figure Legend

Figure 1: A Rif1 (orange) coats late replicating genome (green) and limits the interactions between regions with the same replication timing. **B** In absence of Rif1 late replicating regions of the genome not associated with Lamin B1 change replication timing as well as lose replication-timing specificity of interactions.

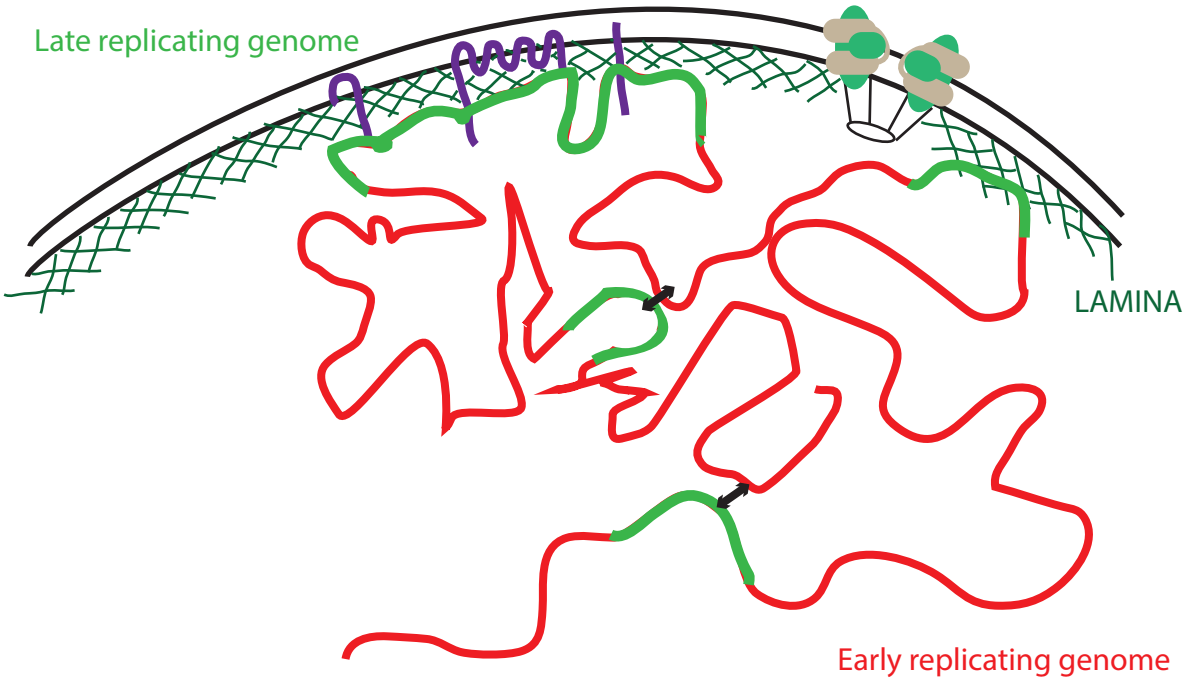
Figure 2: A At the end of mitosis, replication timing determinants are lost together with chromatin organized positions in the nucleus. **B** Re-establishment of replication timing takes place in G1. Rif1 associates with chromatin at the end of telophase-early G1 (Yamazaki et al., 2012), in time to enforce the restriction of 3D contacts between genomic regions within the same replication timing at the TDP. The result is the architectural compartmentalization of the late replicating genome and the consequent creation of domains of high PP1 density. **C** When DDK activity increases at G1/S, late origins are embedded in RADs and surrounded by high concentrations of PP1. Possible physical sequestration and high concentrations of phosphatase inhibit their firing.

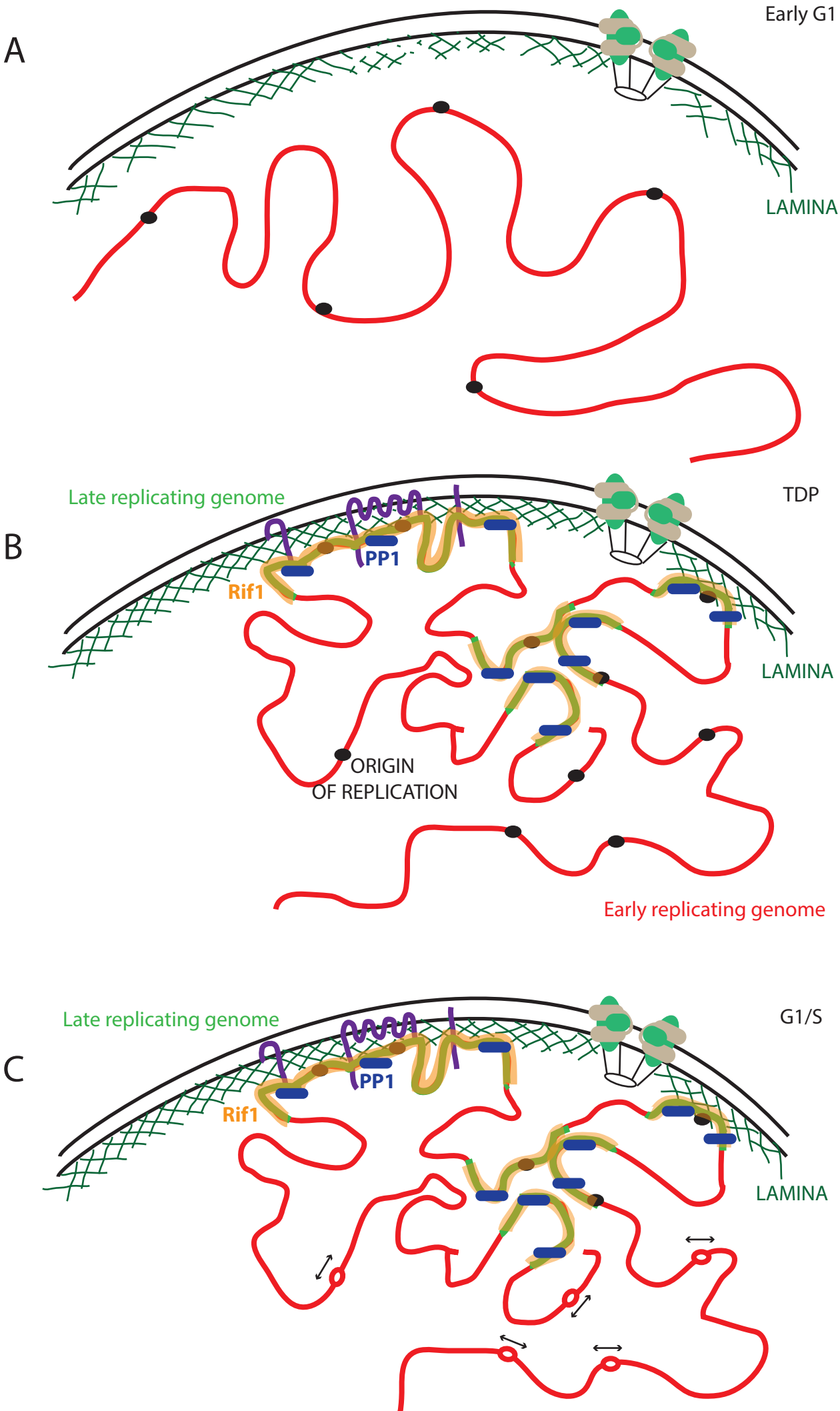
Figure 3: Nuclear architecture and DNA replication timing could be two independent processes connected only by their respective dependence upon Rif1 (Hypothesis 1 and 2). Alternatively, Rif1-dependent organization of nuclear architecture could also influence the firing of replication origins independently of Rif1 (Hypothesis 3).

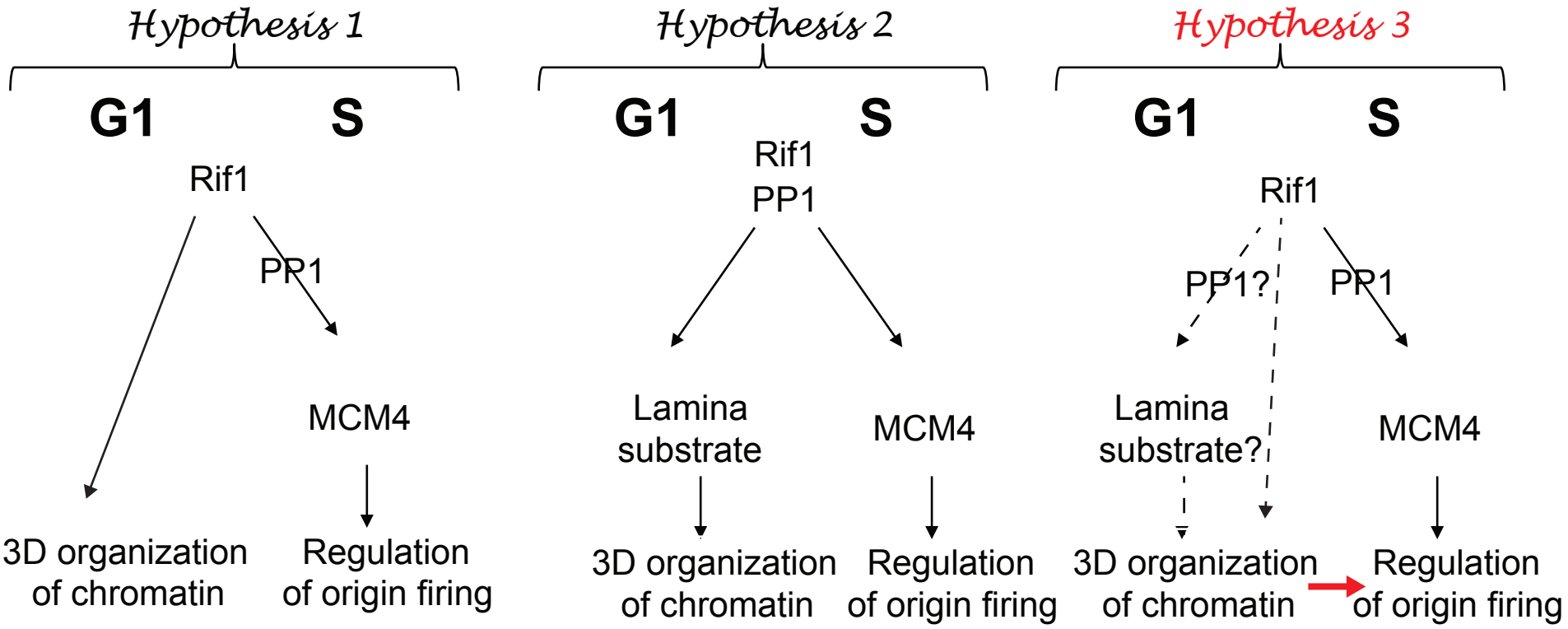
A


Late replicating genome
Rif1
LAMINA
Early replicating genome

B


Late replicating genome
LAMINA
Early replicating genome


A
Early G1
LAMINA
B
Late replicating genome
TDP
PP1
Rif1
LAMINA
ORIGIN
OF REPLICATION
Early replicating genome
C
Late replicating genome
G1/S
PP1
Rif1
LAMINA
Early replicating genome


Hypothesis 1
G1
S
Rif1
PP1
MCM4
3D organization of chromatin
Regulation of origin firing
Hypothesis 2
G1
S
Rif1
PP1
Lamina substrate
MCM4
3D organization of chromatin
Regulation of origin firing
Hypothesis 3
G1
S
Rif1
PP1?
PP1
Lamina substrate?
MCM4
3D organization of chromatin
Regulation of origin firing